METHOD AND SYSTEM FOR IN VITRO DEVELOPMENTAL TOXICITY TESTING

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of Singapore Patent Application No. 201207242-7 filed on 28 September 2012, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The invention relates to a method and system for developmental toxicity testing.

BACKGROUND

[0003] The following discussion of the background to the invention is intended to facilitate an understanding of the present invention. However, it should be appreciated that the discussion is not an acknowledgment or admission that any of the material referred to was published, known or part of the common general knowledge in any jurisdiction as at the priority date of the application.

[0004] Birth defects are one of the leading causes of infant mortality worldwide[1,2,3] and can also result in long-term disabilities and illnesses. Abnormalities during fetal development can be due to either genetic conditions or environmental exposure during pregnancy, especially the first trimester. Therefore, regulatory bodies, such as the US Environmental Protection Agency (EPA), have mandated that environmental agents, such as drugs, chemicals and pesticides should be evaluated for developmental toxicity[4].

[0005] Testing developmental toxicity on animals is limited by cost and ethical issues. Hence, a number of alternative animal embryo or cell-based in *vitro* developmental models have been developed, which include the frog embryo teratogenesis assay (FETAX)[5], the chicken embryo toxicity screening test (CHEST)[6], the micromass (MM) assay using mouse embryonic mesenchymal cells[7], the mouse or rat whole embryo culture (WEC) assay[8], the zebrafish embryo-larva developmental toxicity assay[9], and the mouse embryonic stem cell test (EST)[10].

[0006] According to the European Centre for the Validation of Alternative Methods (ECVAM), only the MM assay, WEC and mouse EST for embryotoxicity testing are scientifically validated and can be considered for regulatory acceptance and application[11-13]. However, there are inter-species variations that cannot adequately explain differences in the molecular regulation of embryonic development between human and animals, or when used in reproductive toxicity testing, generate false negatives with devastating consequences. For instance, the withdrawn drug, Thalidomide, causes developmental deformation in human but not in mouse[14]. This technology aims to provide a *human cell-based developmental model* for research and screening applications in developmental toxicity.

[0007] There have been attempts to replace mouse ESCs used in the EST assay with human embryonic stem cells (hESCs)[16,17], although there have been no successful development of a human EST. The core standard of mouse EST is to evaluate the toxicity of compounds based on their effect on mouse embryonic stem cell (mESC) differentiation to beating cardiomyocytes[10]. By using mouse ES cell line D3 and mouse embryonic fibroblast cell line 3T3, they try to measure inhibition of cytotoxicity (IC_{50}) values for both cell lines, and inhibition of differentiation (ID_{50}) values for mESCs. They further classify the drug compounds into three classes as "non-embryotoxic", "weakly embyotoxic" and "strongly embryotoxic" using validated prediction model. The entire process lasts for 10 days using traditional beating cardiomyocytes monitoring under microscope or 7 days using FACS to check the gene expression of cardiac tissue. One major reason is that hESCs differentiate *in*

vitro into cardiomyocytes at a slower rate and take longer time than mESCs (10-25% after 30 days)[18], making it impossible to count the beating cardiomyocytes on Day 10. Using RT-PCR or immunostaining method, researchers could acquire the data describing the effects of developmental toxins. However, lacking suitable scoring system makes it still unsatisfactory for drug testing application.

[0008] In 2010, Cezar's group showed for the first time the successful classification of drugs into developmental toxins and non-developmental toxins using metabolomics and random forest modeling[19]. However, they just tested those drugs in hESC pluripotency maintenance medium (i.e., mTeSR1 medium) instead of actually differentiating hESC, and they cannot further classify those developmental toxins into weak or strong developmental toxic compounds since only circulating concentration of the drugs was applied in the experiments.

[0009] While the current cell-based MM and EST assays can potentially incorporate human embryonic or pluripotent cells, the developmental process (i.e. cells differentiating into the 3 germ layers) is spontaneous and disorganized in these models. Consequently, they do not provide a sensitive and reliable way of classifying developmental toxins, which includes embryo toxins and teratogens, because the assays are either measuring general cytotoxicity[7] or inhibition of cardiac tissue formation[10], and are too crude to capture an important aspect of developmental toxicity – disruption of differentiation patterns. Current EST models rely on measuring inhibition of cardiomycyte formation by xenobiotics as an indicator of developmental toxicity. Therefore it is not compatible with the intrinsic property of hESCs as hESCs do not form cardiomyocytes readily, unlike mouse ES cells.

[0010] The object of the invention is to ameliorate at least one of the problems mentioned herein.

SUMMARY OF THE INVENTION

[0011] Throughout this document, unless otherwise indicated to the contrary, the terms "comprising", "consisting of", and the like, are to be construed as non-exhaustive, or in other words, as meaning "including, but not limited to".

[0012] The present technology includes an *in vitro* method of developmental toxicity testing comprising the steps of:

a. micropatterning an extracellular matrix;

b. growing embryonic stem cells on the micropatterned extracellular matrix in the presence of a mesoendodermal induction medium for forming a geometrical mesoendoderm structure; and

c. testing for change of the geometrical mesoendoderm structure in a presence or an absence of a test compound wherein (1) a decrease in mesoendodermal cell differentiation and/or (2) a change in morphology of the geometrical mesoendoderm structure in the presence of the test compound compared to cells in the absence of the test compound indicates that the test compound is a developmental toxic agent.

[0013] The use of a mesoendodermal induction agent mimics one of the earliest processes of embryonic development (primitive streak formation). The advantage of the method may be to have more than one quantitative descriptors of developmental toxic effects.

[0014] Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following illustrative drawings of preferred embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Preferred embodiments of the invention will be described, by way of illustrative examples only, with reference to the following drawings, of which:

[0016] **Figure 1**: Cell patterning using polydimethylsiloxane (PDMS) stencil.

[0017] **Figure 2**: Formation of a geometrical mesoendoderm structure coinciding with the shape of the hESC colonies after 3 days of differentiation. (a), (b), (e), (f) are hESC colonies with the area of 785000 μm^2; (c), (d) are hESC colonies with the area of 196250 μm^2. Red: Immunofluorescence staining for mesoendodermal marker Brachyury; Scale bar: 200μm.

[0018] **Figure 3**: Confocal scans of the edge of hESC colony (circle). Red: Brachyury, Blue: DAPI. (a) 3-D reconstruction shows that Brachyury-positive cells localize at the edges of hESC colony, forming a shining "ring"; (b) Z-stack shows that Brachyury-positive cells only localize on the top layer of the cell sheet, and there is a "tube-like" structure beneath the shining "ring". Scale bar: 30μm.

[0019] **Figure 4**: The expression of Brachyury with/without drug treatment after 3 days of differentiation. T expression was mis-localized after treated with 5-fluorouracil (5-FU) (b), valproic acid (VPA) (c) or Thalidomide (d). Scale bar: 100μm.

[0020] **Figure 5**: Asymmetrical Ratio Calculation. First acquire the radii of the ring (red color in a) & b)) using untreated hESC colonies, then apply this ring as a mask to hESC colonies treated with teratogens (c), calculate the two kinds of Asymmetrical Ratios independently and compare the relative change.

[0021] **Figure 6**: Teratogen-induced change in Asymmetry Ratio relative to untreated control. a) Asymmetrical Ratio value with or without teratogen treatment. b) The relative Asymmetrical Ratio change after teratogen treatment compared with untreated control. For 0.05 μg/ml 5-FU, the Asymmetrical Ratio decreased by 46.7%, from 3 to 1.6; for 0.1 mM VPA and 0.8 mM Thalidomide, the Asymmetrical Ratio decreased by ~70%, from 3 to ~0.9

[0022] **Figure 7**: Images of geometrical mesoendoderm structures formed from micropatterning different hESC cell lines. **(A)**: Micropatterned H9 hESCs after 3 days of differentiation. **(B)**: Micropatterned H1 hESCs after 3 days of differentiation

[0023] **Figure 8**: The geometrical mesoendoderm structure was formed by spatially patterned differentiation followed by coordinated morphogenetic cell movement. **(A)**: The phase and fluorscence images of the micropatterned hESC (μP-hESC) colony on day1-day3. The mesoendoderm (T^+) cells were spatially patterned at the colony edge on day 1, then displaced to ~150-200μm away from the colony edge on day 3. **(B)**: Spatial patterning of other mesoendoderm markers after 3 days of differentiation. Wnt3a, Fgf8, Cripto1 (green) colocalized with T (red) in the μP-hESC colony. **(C)**: Transcription levels of epithelial-mesochymal transition (EMT) markers around the μP-hESC colony edge and center. Cells near the edge of the μP-hESC colony have a higher expression levels of EMT markers such as Snail and Vimentin compared with cells in the colony centre. Brachyury (T), Wnt3a, Fgf8 and Cripto1 are all mesoendoderm markers. **(D)**: The montage of the 3-day live imaging of the μP-hESC colony under 10X objective showed the movement of the cells during the 3-day differentiation. **(E)**: Kymograph analysis along the yellow line of the colony on Fig. 1D during the 3-day live imaging window. Cells near the colony edge showed random cell migration on day 2, but underwent directed collective cell movement on day 3.

[0024] **Figure 9**: Developmental toxic drugs affected the physical morphology / localization of the mesoendoderm structure in the μP-hESC model. **(A)**: Kymographs of the μP-hESC colony under drug treatment. The trajectories of the collective cell migration under developmental toxic drug treatment (Thalidomide & VPA) were disrupted during day2-3, while the collective cell migration under non-developmental toxic drug treatment (Penicillin G) was unaffected. **(B)**: Mesoendoderm marker T localization with/without drug treatment on day3. The localization of the mesoendoderm motif (T^+ region) was misplaced when treated with Thalidomide or VPA, while remained unchanged when treated with negative control drug Penicillin G. Scale bar: 100μm.

[0025] **Figure 10**: PCA results of the dose-dependent developmental toxicity responses caused by developmental toxic drugs. **(A-C)**: The spatial distribution of

mesoendoderm motif within the μP-hESC colonies were significantly disrupted when treated with Retinoid acid (RA, >=0.0036 μg/ml), Thalidomide (>=300 μM), or VPA (>= 0.8 mM). **(D)**: The drug treatment of Penicillin G could not significantly disrupt the spatial distribution of the mesoendoderm motif within the μP-hESC colonies even when the concentration was 1000 μg/ml.

[0026] **Figure 11**: Drug classification results based on PCA and cytotoxicity test (MTS) results. **(A)**: The effective concentration (the lowest concentration of the drug which can significantly disrupted the spatial localization of the mesoendoderm motif in the μP-hESC models) of RA (EC_{RA}) was ~0.0036 μg/ml, which was not toxic either to hESC cell line H9 or to adult human dermal fibroblasts (aHDFs). Therefore RA is a developmental toxic drug which is teratogenic. **(B)**: The $EC_{Thalidomide}$ was ~300 μM, which was not toxic to aHDFs but toxic to H9 cells (>50% survival). Therefore Thalidomide is a developmental toxic drug which is both teratogenic and embryotoxic. **(C)**: The EC_{VPA} was ~0.8 mM, which was a little toxic to aHDFs but very toxic to H9 cells (~ 20% survival). Therefore VPA is a developmental toxic drug which is very embryotoxic (which means very toxic to hESCs). **(D)**: The $EC_{Penicillin\ G}$ was above 1000 μg/ml (which is the highest concentration tested for most developmental toxicity testing platforms). Therefore Penicillin G is a non-developmental toxic drug.

DETAILED DESCRIPTION

[0027] An *in vitro* method of developmental toxicity testing is described comprising the steps of

micropatterning an extracellular matrix;

growing embryonic stem cells on the micropatterned extracellular matrix in the presence of a mesoendodermal induction medium for forming a geometrical mesoendoderm structure; and

testing for change of the geometrical mesoendoderm structure in the presence or absence of a test compound wherein (1) a decrease in mesoendodermal cell differentiation and/or (2) a change in morphology of the geometrical mesoendoderm structure in the presence of the test compound compared to cells in the absence of the test compound indicates that the test compound is a developmental toxic agent.

[0028] Preferably, the change in morphology of the geometrical mesoendoderm structure comprises a change in shape and/or location of the geometrical mesoendoderm structure.

[0029] Preferably, the mesoendodermal induction medium comprises a mixture of activin amino acid, bone morphogenic protein, and fibroblast growth factor 2. However, any medium known in the art to initiate mesoendodermal differentiation would be suitable. The mixture may further comprises vascular endothelial growth factor.

[0030] Activin may be any isolated, regenerative or synthetic activin protein known in the art. In mammals, four beta subunits of activin have been described, called activin β_A, activin β_B, activin β_C and activin β_E. Preferably, activin A comprising a dimer of activin β_A is used in the mesoendodermal induction medium. Preferably activin beta A chain has an amino acid sequence of SEQ ID NO. 1:

MPLLWLRGFLLASCWIIVRSSPTPGSEGHSAAPDCPSCALAALPKDVPNSQPEMVE
AVKKHILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVEIEDDIGRRAEM
NELMEQTSEIITFAESGTARKTLHFEISKEGSDLSVVERAEVWLFLKVPKANRTRTKV
TIRLFQQQKHPQGSLDTGEEAEEVGLKGERSELLLSEKVVDARKSTWHVFPVSSSI
QRLLDQGKSSLDVRIACEQCQESGASLVLLGKKKKKEEEGEGKKKGGGEGGAGAD
EEKEQSHRPFLMLQARQSEDHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWND
WIIAPSGYHANYCEGECPSHIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPT
KLRPMSMLYYDDGQNIIKKDIQNMIVEECGCS

[0031] Bone morphogenic proteins (BMP) are a group of growth factors also known as cytokines or as metabologens. There are twenty known BMPs. Any isolated regenerative or synthetic BMP may be suitable. Preferably BMP4 is used in the mesoendodermal induction medium. Preferably, human BMP4 has an amino acid sequence of SEQ ID NO. 2:

MIPGNRMLMVVLLCQVLLGGASHASLIPETGKKKVAEIQGHAGGRRSGQSHELLRD
FEATLLQMFGLRRRPQPSKSAVIPDYMRDLYRLQSGEEEEEQIHSTGLEYPERPAS
RANTVRSFHHEEHLENIPGTSENSAFRFLFNLSSIPENEVISSAELRLFREQVDQGPD
WERGFHRINIYEVMKPPAEVVPGHLITRLLDTRLVHHNVTRWETFDVSPAVLRWTR
EKQPNYGLAIEVTHLHQTRTHQGQHVRISRSLPQGSGNWAQLRPLLVTFGHDGRG
HALTRRRRAKRSPKHHSQRARKKNKNCRRHSLYVDFSDVGWNDWIVAPPGYQAF
YCHGDCPFPLADHLNSTNHAIVQTLVNSVNSSIPKACCVPTELSAISMLYLDEYDKVV
LKNYQEMVVEGCGCR

[0032] Fibroblast growth factors (FGF) are a group of growth factors. There are 22 known FGFs in humans which are all structurally related but only those FGFs that bind heparin, for example FGF2would be suitable for use in the mesoendodermal induction medium. Preferably, human FGF2 has an amino acid sequence of SEQ ID NO. 3:

MVGVGGGDVEDVTPRPGGCQISGRGARGCNGIPGAAAWEAALPRRRPRRHPSVN
PRSRAAGSPRTRGRRTEERPSGSRLGDRGRGRALPGGRLGGRGRGRAPERVGG
RGRGRGTAAPRAAPAARGSRPGPAGTMAAGSITTLPALPEDGGSGAFPPGHFKDP
KRLYCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGVVSIKGVCANRYLAM
KEDGRLLASKCVTDECFFFERLESNNYNTYRSRKYTSWYVALKRTGQYKLGSKTGP
GQKAILFLPMSAKS

[0033] In one embodiment the mesoendodermal induction medium further comprises vascular endothelial growth factor (VEGF). Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. The broad term 'VEGF' covers a number of proteins known in the

art. VEGF may be used in the mesoendodermal induction medium. Preferably, human VEGF has an amino acid sequence of SEQ ID NO. 4:

MNFLLSWVHWSLALLLYLHHAKWSQAAPMAEGGGQNHHEVVKFMDVYQRSYCHP
IETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTEESNITMQIMRIKPHQ
GQHIGEMSFLQHNKCECRPKKDRARQEKKSVRGKGKGQKRKRKKSRYKSWSVYV
GARCCLMPWSLPGPHPCGPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLELN
ERTCRCDKPRR

[0034] In one embodiment the micropatterning is achieved by fabricating a stencil of a polydimethylsiloxane (PDMS) sheet having a plurality of geometric shapes cut into the PDMS sheet the PDMS stencil is sealed into a culture container, the matrix is coated over the PDMS stencil within the culture container such that when the PDMS stencil is removed only cells growing in the plurality of geometric shapes remain in the culture container. Preferably, the culture container is a petri dish or a well in a 96 well plate, however any container that will hold a matrix to grow embryonic stem cells would be suitable.

[0035] In another embodiment the micropattern is formed by applying a plurality of growth factor gradients on the matrix. The plurality of growth factor gradients may be printed onto the matrix as a plurality of geometric shapes using methods such as microcontact printing, microfluidic patterning, inkjet printing or any other method known in the art to print a pattern onto a matrix.

[0036] In a one embodiment the plurality of geometric shapes are all the same size and shape.

[0037] In another embodiment the plurality of geometric shapes vary in size or vary in shape.

[0038] The plurality of geometric shapes may be any shape for example circular, square, semi-circular, rectangular or any shape required.

[0039] Preferably the culture media comprises a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. This is known as Matrigel™ (BD Biosciences) or Cultrex BME ™ (Trevigen Inc). Other suitable culture mediums able to support embryonic cell growth and differentiation would be suitable such as gelatin, laminin or synthetic peptides known in the art.

[0040] The method of testing can be used with any embryonic stem cells but preferably the embryonic stem cells are human embryonic stem cells (hESC).In one embodiment the embryonic stem cells are seeded at a density of 4 million cells/ml.

[0041] Preferably the testing for mesoendodermal cell differentiation is achieved by incubating the cells with antibody against a mesoendodermal marker and imaging the cells for detection of the marker.

[0042] Preferably a variation in mesoendodermal cell differentiation at an outer perimeter of the micropattern in the presence of the test compound compared to in the absence of the test compound indicates that the test compound is a developmental toxic agent.

[0043] Our proposed technology provides a novel approach of assessing for chemical- or drug-induced developmental toxicity in human stem cells. Our technology recapitulates spatial patterning of mesoendoderm differentiation, which marks the earliest differentiation event in a localized region of a gastrulating embryo. We achieve this by patterning human stem cells onto extracellular matrix (ECM) islands of specified geometries to impose a mechanical gradient on the human embryonic stem cell (hESC) colony. Cells at the geometry edge of the colony are predisposed to differentiate into mesoendoderm. The micropatterned hESC colonies can generate mesoendoderm patterns corresponding to the geometrical shape of the colony. This differentiation pattern can be disrupted by known teratogents, such as valproic acid and thalidomide, at non-cytotoxic concentrations. The differentiation pattern arising from the endogenous mechanical stress in the micropatterned human stem colony can be quantified via image processing, and provides a quantitative

measurement for the developmental toxicity of different chemicals and drugs.

[0044] Spatially patterned hESC differentiation arising from endogenous mechanical gradient in micropatterned cell colony.

[0045] Our technology can complement ToxCast, which uses computational modeling to predict liver and developmental toxicity, by providing hESC experimental data to refine the v-Embryo database.

[0046] The micropatterned platform can be used as an experimental tool for studying developmental biology.

[0047] The advantages of our technology includes the following:-

- Technique works with human embryonic or pluripotent stem cells
  - Our proposed technique has been shown to work with human ES cells.
- Shorter time to establish model for experimentation/drug testing.
  - 3 days instead of 7/10 days in mouse EST.
- Measures disruption of differentiation pattern instead of changes in level of differentiation, which is difficult to benchmark.
- By measuring disruption of differentiation pattern, we can quantitatively acquire of the Asymmetrical Ratio change, based on which a clear classification of the drugs could be made. However, in current models which measure changes in the level of differentiation of hESC, since even the expression changes of different genes that belong to the same lineage can be quite different from one another under the same conditions.
- Discriminates between non-specific cytotoxicity and disruption of lineage-specified differentiation.
  - Our technology generates hESC differentiation patterns and measures disruption of these morphological patterns as the assay read-out for developmental toxicity testing. Since we do not measure cell viability, our measurement is not confounded with general cytotoxicity, which also affects cell viability.

- Compatible with high-throughput and high-content image analysis systems.
  - For example, we can adapt our model to a 96-well plate format, which could make matrix coating much easier and less time-consuming. After seeding hESC in the 96-well plate, all the following sample preparation procedures including daily medium change and immunofluorescence staining could be automated using a JANUSTM automated liquid handling system (Perkin Elmer) (customized by BioLaboratories, Singapore). Furthermore, images could be acquired using CellomicsR ArrayScanR VTI HCS Reader (Thermo Scientific) to significantly improve imaging speed. Finally, for data analysis, we could just modify the program to make it capable of doing serial Asymmetrical Ratio calculations in MATLAB software.

[0048] Instead of controlling spatial differentiation using mechanical stress, we can also directly pattern growth factors to induce localized differentiations, forming a growth factor gradient rather than a sparcial gradient which may also be applicable for developmental toxicity testing. In this example the micropatterning is made by varying the amount of mesoendodermal induction medium on a culture medium. Growth factor patterning using multi-step micropatterning or microfluidics, may be more difficult to handle but would still work.

Example

Materials & methods

[0049] *Cell patterning*

In order to generate gradients of mechanical stress, circle or square shapes of Matrigel matrix with two different sizes (785000 μm2 or 196250 μm2) were designed using AutoCAD software. Then polydimethylsiloxane (PDMS) stencils were fabricated by using a laser-cutter (Universal Laser System) to cut the designated shape patterns on a 127 μm thick PDMS sheet (Specialty Silicone Products Inc.) and then bonding it to a laser-cut, 2 mm thick PDMS gasket. For cell patterning, the PDMS stencil was

first sealed into a 60mm petri dish by adding 200μl 70% ethanol and dried in the tissue culture hood, then Matrigel™ (BD Biosciences) matrix was coated by at least 1 hour incubation with the Matrigel™ solution (Figure 1). Thereafter cells were seeded at a density of 4 million cells/ml. After 1 hour incubation for cell attachment, the stencil and excess unattached cells were removed. The surface was then passivated with 0.5% pluronic acid for 10 mins before 3-time washing. The acquired patterned hESC were incubated overnight in mTesR1 maintenance medium (StemCell Technologies) for stabilization and the mesoendoderm induction was started the next day.

[0050] *Mesoendoderm induction*

The differentiation of patterned hESC colonies were induced by mesoendoderm induction medium, which comprises of 100 ng/ml Activin, 25 ng/ml BMP4 and 10 ng/ml FGF2 in basal serum-free APEL™ medium (STEMdiff™, StemCell Technologies). For developmental toxicity testing, the patterned hESC colonies were cultured in mesoendoderm induction medium together with the test chemical. The induction lasted 3 days and the medium is half changed daily with/without the test chemical.

[0051] *Immunofluorescence staining, Imaging & data analysis*

Samples were fixed on Day 3 of differentiation. Immunofluorescence staining was done to check the expression distribution of early mesoendodermal marker Brachyury. Image acquisition was done under 10X objective using Olympus fluorescence microscope and Asymmetrical Ratio was calculated using MATLAB programming (Figure 5).

Results

[0052] *Spatial asymmetrical mesoendormal differentiation pattern formed in vitro*

After 3 days of culture in mesoendoderm induction medium, the spatial localization of

mesoendoderm in the colonies were coincidental with the geometrical shapes of the colonies, forming a "ring-like" structure (Figure 2 & Figure 3). In other words, after 3 days of differentiation, there is formation of a geometrical mesoendoderm structure coinciding with the shape of the hESC colonies. This "ring-like" structure had a unique 3-D structure, with Brachyury-positive cells sitting on the top layer of the cell sheet, and a "tube-like" structure forming beneath the shining "ring". This phenomenon indicated that localized cell differentiation and morphogenetic movements did occur in our system, which are also concurrent and inevitable in human embryogenesis.

[0053] *Spatial asymmetrical pattern disruption by teratogens*

Three known teratogens (0.1 mM Valproic acid (VPA), 0.05ug/ml 5-Fluorouracil (5-FU), and 0.8mM Thalidomide) were tested using this model. Mesoendoderm induction medium together with the drug was added into our model and incubated for 3 days before the samples were fixed for immunofluorescence staining of Brachyury. Half of the medium was changed daily with the drug. An untreated control which was only cultured in mesoendoderm induction medium without any drug was also done. Results showed that all of the three drugs could disrupt the original asymmetrical differentiation pattern, i.e. destroy the "ring" formation (Figure 4). In order to measure the disruption, we used Asymmetrical Ratio as a parameter to evaluate the asymmetrical patterns with or without drug treatment, which indicate the average intensity ratio of "ring" area to "off-ring" area (Figure 5). Results showed that for untreated controls, the average Asymmetrical Ratio was around 2.5-3.5 (Figure 6). When treated with teratogens, Asymmetrical Ratio values were significantly decreased by developmental toxicity effects. For 0.05 μg/ml 5-FU, the Asymmetrical Ratio was decreased by 46.7%, from 3 to 1.6; and for 0.1mM VPA and 0.8mM Thalidomide, the Asymmetrical Ratio was decreased by ~70%, from 3 to ~0.9. These results implied that our model was quite sensitive to different teratogen treatments (Figure 6), the effects of which could be quantitatively measured and used for drug classification and scoring.

[0054] Geometrical shaped stencil

Stencils of different geometrical shapes (rectangle, circle, semi-circle, square) were made to determine if PS induction was affected with H9 hESCs

1. 60mm PS dish was used,
2. The stencil was sealed onto the 60mm PS dish with ethanol,
3. It was dried under UV light

2 plates were made

Plate 1: had a mixture of large and small shapes

Plate 2: had large sized shapes only

1. Warm mTeSR 1, accutase and DMEM/F12
2. Supplement 3ml TeSR1 with 10um ROCKi (add 2ul of 5mM stock per medium).
3. Remove differential regions,
4. Wash culture once with DMEM/F12,
5. Add 1ml of accutase per 60mm dish and incubate at 37^0C for 5-10 min,
6. Triturate gently to break colonies into single cells
7. Transfer cells into 15ml tube,
8. Rinse plate with at least 5ml of DMEM/F12 per 1 ml of accutase and collect medium into the 15 ml tube,
9. Centrifuge cells at 1000 rpm, 3-5 min,

10. Remove cells and resuspend in 1.5 ml of supplemented mTeSR1 per stencil (use 3 x 6 wells for 2X stencils),

11. Aspirate cell suspension, wash once with DMEM/F12,

12. Add 3ml of DMEM/F12 to area surrounding stencil. Use a foforcep to gently remove stencil. Remove DMEM/F12,

13. Add 3 ml of APEL medium + 1ng/ml Activin A, 25 ng/ml BMP4 and 10ng/ml of FGF2,

14. Change half of volume after 2 days (1.5 ml),

15. Fix and perform immunostaining.

Immunostaining

Samples were fixed for 20 min in 3.7% paraformaldehyde, and permeabilized for 15 min with 0.5% Triton X-100 in PBS. After overnight incubation at 4 °C in blocking buffer (2% BSA and 0.1% Triton X-100 in TBS buffer), they were incubated overnight at 4 °C with primary antibodies (5-10 μg/ml in blocking buffer). The primary antibody used was goat-anti-Brachyury (10 μg/ml, AF2085, R&D systems). The samples were washed 5 times with 15 min interval before adding the Alexa Fluor dye-conjugated secondary antibodies (1:1000, Molecular Probes). After 1 hr incubation at room temperature, samples were washed for 5 times with 15 min interval and counter-stained with Hoechst 33342 (10 μg/ml, Molecular Probes) for 5 min. After that, samples were washed 3 times with PBS and then mounted using Fluorsave™ (Calbiochem).

[0055] Figure 7 shows images of geometrical mesoendoderm structures formed from micropatterning different hESC cell lines.. Two hESC lines (H9 and H1) that were micropatterned into different shapes and sizes generated a geometrical mesoendoderm structure after 3 days of mesoendoderm differentiation. Fig 7A shows micropatterned H9 hESCs after 3 days of differentiation and Fig 7B shows

micropatterned H1 hESCs after 3 days of differentiation. Left panel shows phase contrast images; right panel shows immunostaining of mesoendoderm marker, Brachury (T). Scale bars = 200 μm.

[0056] *Spatially patterned differentiation followed by coordinated morphogenetic cell movement*

Figure 8 shows the characterization of the geometrical mesoendoderm structural motif. The geometrical mesoendoderm structure was formed by spatially patterned differentiation followed by coordinated morphogenetic cell movement. Fig 8A shows the phase and fluorescence images of the micropatterned hESC (μP-hESC) colony on day1-day3 (D1-D3). The mesoendoderm (T^+) cells were spatially patterned at the colony edge on day 1, then displaced to ~150-200μm away from the colony edge on day 3. The geometrical structural motif is formed by (1) spatially patterned mesoendoderm differentiation followed by (2) coordinated cell movement (Fig 8A).

[0057] Fig 8B shows spatial patterning of other mesoendoderm markers after 3 days of differentiation; Wnt3a, Fgf8, Cripto1 (green) colocalized with T (red) in the μP-hESC colony. Fig 8C shows transcription levels of epithelial-mesochymal transition (EMT) markers around the μP-hESC colony edge and center. Cells near the edge of the μP-hESC colony have higher expression levels of EMT markers such as Snail and Vimentin compared with cells in the colony centre. Brachuary (T), Wnt3a, Fgf8 and Cripto1 are all mesoendoderm markers. Spatially patterned differentiation was indicated by the localized expression of mesoendoderm markers, Bachyuary, Fgf8, Cripto1 and Wnt3a, (Fig 8B); and epithelial-mesenchymal transition activity (Fig 8C).

[0058] Fig 8D shows the montage of the 3-day live imaging of the μP-hESC colony under 10X objective which showed the movement of the cells during the 3-day differentiation. Fig 8E shows a kymograph analysis along the yellow line of the colony on Fig. 1D during the 3-day live imaging window. Cells near the colony edge showed random cell migration on day 2, but underwent directed collective cell movement on

day 3. Spatially localized differentiated cells (at edge of stem cell colony) underwent coordinated cell movement to form a 3D-like structural motif as observed by live imaging (Fig 8D). The trajectory of the coordinated cell movement can be seen from a space-time plot (kymograph) of the differentiating stem cells (Fig 8E).

[0059] *Developmental toxic drugs affected physical morphology/localization of mesoendoderm structure in μP-hESC model*

Figure 9 shows alterations to the geometrical mesoendoderm structural motif caused by known developmental toxins. Fig 9A shows kymographs of the μP-hESC colony under drug treatment. The trajectories of the collective cell migration under developmental toxic drug treatment (Thalidomide & VPA) were disrupted during day2-3, while the collective cell migration under non-developmental toxic drug treatment (Penicillin G) was unaffected. In other words, known developmental toxins, valproic acid and thalidomide, altered the trajectory of cell movement, whereas a non-developmental toxin (Penicillin G) did not (Fig 9A). Fig 9B shows Mesoendoderm marker T localization with/without drug treatment on day3. The localization of the mesoendoderm motif (T^+ region) was misplaced when treated with Thalidomide or VPA, while remained unchanged when treated with negative control drug Penicillin G. Scale bar: 100μm. In other words, valproic acid and thalidomide also changes the morphology of the mesoendoderm structural motif, whereas untreated sample or penicillin G-treated sample did not (Fig 9B).

[0060] *Dose-dependent developmental toxicity responses caused by developmental toxic drugs*

Figure 10 shows that the proposed method is sensitive enough to detect different severity of drug-induced effects at different concentrations. Alterations to the different morphological features of the mesoendoderm structural motif were quantitatively measured by image processing at different concentrations of drugs. These morphological features at different drug concentrations were analyzed with principal component analysis (PCA). A data point represents each morphological feature; the

data point color represents the drug concentration used in the treatment. Fig 10A-C shows the spatial distribution of mesoendoderm motif within the μP-hESC colonies were significantly disrupted when treated with Retinoid acid (RA, >=0.0036 μg/ml), Thalidomide (>=300 μM), or VPA (>= 0.8 mM). Fig 10D shows the drug treatment of Penicillin G could not significantly disrupt the spatial distribution of the mesoendoderm motif within the μP-hESC colonies even when the concentration was 1000 μg/ml. For known developmental toxins i.e., (A) retinoic acid (RA), (B) thalidomide, (C) valproic acid (VPA), morphological features from the same drug concentration segregated into distinct clusters, and (D) For a non-developmental toxin (Penicillin G), morphological features from different drug concentration treatment cannot be segregated.

Data collection process:

1. Treat our μP-hESC colonies with 3 different concentrations of each drug and fix the samples on day 3 together with the untreated normal control samples. Acquire T immunofluorescence images of samples under Olympus fluorescence microscope.

2. Process all the T fluorescence images using Matlab to extract all the relevant morphological features of the samples representing the spatial distribution of the mesoendoderm motif (T^+ positive region in the images).

Primary component analysis (PCA):

Perform PCA in Matlab on all the extracted features across all the samples for each drug together with the untreated controls and plot the PCA results.

[0061] *Drug classification by their developmental toxicity effects*

Figure 11 shows that the proposed method can be combined with viability assays on adult and embryonic cells to correctly classify the drugs by their developmental toxicity effects. Fig 11A shows the effective concentration (the lowest concentration of the drug which can significantly disrupted the spatial localization of the

mesoendoderm motif in the μP-hESC models) of RA (ECRA) was ~0.0036 μg/ml, which was not toxic either to hESC cell line H9 or to adult human dermal fibroblasts (aHDFs). Teratogenic drugs alter morphology of mesoendoderm structural motif at concentrations that did not affect viability of both embryonic and adult cells (e.g., retinoic acid (Fig 11A). Therefore RA is a developmental toxic drug which is teratogenic.

[0062] When the effective drug concentration resulting in alteration to the structural motif overlaps with the drug concentration reducing embryonic cell viability, the drug is embryotoxic (e.g. thalidomide and valproic acid (Fig 11B-C)). Fig 11B shows the $EC_{Thalidomide}$ was ~300 μM, which was not toxic to aHDFs but toxic to H9 cells (>50% survival). Therefore Thalidomide is a developmental toxic drug which is both teratogenic and embryotoxic. Fig 11C shows the EC_{VPA} was ~0.8 mM, which was a little toxic to aHDFs but very toxic to H9 cells (~ 20% survival). Therefore VPA is a developmental toxic drug which is very embryotoxic (which means very toxic to hESCs).

[0063] When the mesoendoderm structural motif was not affected at drug concentrations where one observes decrease in cell viability of adult and embryonic cells, the drug is non-developmental toxic (e.g. penicillin G (Fig 11D)). Fig 11D shows the $EC_{Penicillin\ G}$ was above 1000 μg/ml (which is the highest concentration tested for most developmental toxicity testing platforms). Therefore Penicillin G is a non-developmental toxic drug.

[0064] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. The invention includes all such variation and modifications. The invention also includes all of the steps, features, formulations and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[0065] Each document, reference, patent application or patent cited in this

text is expressly incorporated herein in their entirety by reference, which means that it should be read and considered by the reader as part of this text. That the document, reference, patent application or patent cited in this text is not repeated in this text is merely for reasons of conciseness.

[0066] Any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention.

[0067] The present invention is not to be limited in scope by any of the specific embodiments described herein. These embodiments are intended for the purpose of exemplification only. Functionally equivalent products, formulations and methods are clearly within the scope of the invention as described herein.

[0068] The invention described herein may include one or more range of values (e.g. size, concentration etc). A range of values will be understood to include all values within the range, including the values defining the range, and values adjacent to the range which lead to the same or substantially the same outcome as the values immediately adjacent to that value which defines the boundary to the range.

[0069] Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. It is also noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect

a basic or novel characteristic of the invention.

[001] Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

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CLAIMS

1. An *in vitro* method of developmental toxicity testing comprising the steps of:

a. micropatterning an extracellular matrix;

b. growing embryonic stem cells on the micropatterned extracellular matrix in the presence of a mesoendodermal induction medium for forming a geometrical mesoendoderm structure; and

c. testing for change of the geometrical mesoendoderm structure in a presence or an absence of a test compound wherein (1) a decrease in mesoendodermal cell differentiation and/or (2) a change in morphology of the geometrical mesoendoderm structure in the presence of the test compound compared to cells in the absence of the test compound indicates that the test compound is a developmental toxic agent.

2. The method of claim 1, wherein the mesoendermal induction medium comprises Activin amino acid, bone morphogenic protein, and fibroblast growth factor.

3. The method of claim 2, wherein the mesoendermal induction medium further comprises vascular endothelial growth factor.

4. The method of claim 1, wherein the change in morphology of the geometrical mesoendoderm structure comprises a change in shape and/or location of the geometrical mesoendoderm structure.

5. The method of claim 1, wherein the micropatterning is achieved by fabricating a stencil of a polydimethylsiloxane (PDMS) sheet having a plurality of geometric shapes cut into the PDMS sheet the PDMS stencil is sealed into a culture container, the matrix is coated over the PDMS stencil within the culture container such that when the PDMS stencil is removed only cells growing in the plurality of geometric shapes remain in the culture container.

6. The method of claim 1, wherein the micropattern is formed by applying a plurality of growth factor gradients on the matrix.

7. The method of claim 6, wherein the plurality of growth factor gradients are printed as a plurality of geometric shapes onto the matrix.

8. The method of claim 5 or 7, wherein the plurality of geometric shapes is all the same size and shape.

9. The method of claim 5 or 7, wherein the plurality of geometric shapes varies in size or shape.

10. The method of any one of claims 5, or 7 to 9, wherein the plurality of geometric shapes is circular.

11. The method of any one of claims 5 or 7 to 9, wherein the plurality of geometric shapes is square.

12. The method of claim 5 or 7, wherein the matrix comprises a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells.

13. The method of claim 1, wherein the embryonic stem cells are human embryonic stem cells (hESC).

14. The method of claim 1 or 13, wherein the embryonic stem cells are seeded at a density of 4 million cells/ml.

15. The method of claim 1, wherein testing for mesoendodermal cell differentiation is achieved by incubating the cells with antibody against a mesoendodermal marker and imaging the cells for detection of the marker.

16. The method of claim 1, wherein a variation in mesoendodermal cell differentiation at an outer perimeter of the micropattern in the presence of the test compound compared to in the absence of the test compound indicates that the test compound is a developmental toxic agent.

SEQUENCE LISTING

<110> AGENCY FOR SCIENCE TECHNOLOGY AND RESEARCH
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<120> METHOD AND SYSTEM FOR IN VITRO DEVELOPMENTAL TOXICITY TESTING

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